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REVIEW

Cationic liposome-mediated gene transfer

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Direct gene transfer for the treatment of human diseases requires a vector which can be administered efficiently, safely and repeatedly. Cationic liposomes represent one of the few examples that can meet these requirements. Currently, more than a dozen cationic liposome formulations have been reported. These lipsomes bind and condense DNA spontaneously to form complexes with high affinity to cell membranes. Endocytosis of the complexes followed by disruption of the endosomal membrane appears to be the major mechanism of gene delivery. The effectiveness and safety of this DNA delivery method has been established in many studies Based on these results, two human gene therapy clinical trials using cationic liposomes have been conducted and more trials will be started in the near future. The simplicity, efficiency and safety features have rendered the cationic liposome an attractive vehicle for human gene therapy.

Keywords: cationic liposome; DNA; delivery; expression; gene therapy; transfection

Introduction

Many current gene therapy approaches concentrate on reimplanting genetically engineered cells back into the host to achieve long-term expression of a desired gene (for reviews see References 1 and 2). Although *ex vivo* gene delivery is effective, and successful clinical trials have been reported, the application of this approach is limited by the facts that it may only apply to certain types of stem or somatic cells, and that it involves complicated, time consuming and personalized procedures. On the other hand, an approach that allows direct introduction of a therapeutic gene into the target tissue or organ offers a simple and potentially cost-effective alternative, and may have the potential to be used in a variety of types of cells and tissues. Although a few types of cells and tissue, such as muscle,[3] thyroid gland,[4] certain types of tumors,[5] and liver cells,[6] can be transfected by direct injection of naked DNA, the rest of the body is quite resistant to the treatment, unless a carrier is used. Developing DNA delivery technology has been one of the important aspects in the field of gene therapy research.

A number of methods have been used for delivery and expression of foreign genes *in vitro* and *in vivo*. These include chemical methods (calcium phosphate precipitation, DEAE-dextran, polybrene, neutral or anionic liposomes, cationic liposomes and targeted polylysine conjugates etc.), physical methods (microinjection, electroporation and biobalistics) and biological methods (viral vectors).[7] Practically, an ideal gene delivery vector should have the following characteristics: (1) protecting and delivering DNA into cells efficiently, preferably with specificity toward a particular cell type; (2) non-toxic and non-immunogenic; and (3) easy to produce in large quantity. To date, none of the vector systems can meet all these requirements. Adenovirus, for example, is a highly efficient vector for gene transfer and can transiently infect cells of different types. Engineered adenovirus is believed to be relatively safe for the host.[8,9] Compared with other recombinant viral vectors, adenovirus is relatively easy to produce in large quantity. However, recent preclinical and clinical trials have raised serious concerns about its immunogenicity. Treatment related inflammation, production of neutralizing antibodies and virus specific cytotoxic T lymphocyte (CTL) response in the host may prevent this viral vector from being used at high doses or administered repeatedly.[10] Retrovirus and adeno-associated virus (AAV) mediate efficient and stable transfection to dividing and possibly nondividing cells.[11,12] However, relatively low viral titers have been the major technical limitation for both systems

The safety concerns and the difficulty of obtaining a large quantity of the recombinant viral vector have prompted the search for efficient, nonimmunogenic or poorly immunogenic and easy-to-prepare nonviral vector systems. Among them, cationic liposomes and targeted polylysine conjugates are the most

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promising.[13,14] The field of cationic liposome-mediated gene delivery has been pioneered by Felgner and co-workers.[15] Cationic liposomes form complexes with DNA through charge interactions. DNA in the form of a complex is protected from degradative activities. DNA–liposome complexes bind to the negatively charged cell surface due to the presence of excess positive charges in the complex. The nature of nonspecific interaction results in efficient transfection of many cell types. Relatively high transfection efficiency comes from the intrinsic membrane rupturing capability of cationic liposome as a result of destabilizing the endosome and/or plasma membrane. The safety and effectiveness of this gene delivery approach has been demonstrated *in vitro* and *in vivo* in many studies,[16] including two clinical trials.[17,18] In this review, we intend to summarize some of the recent progress in the following aspects concerning cationic liposome-mediated gene transfer: (1) currently available cationic liposome formulations and their performance; (2) mechanism of cationic liposome-mediated gene transfer; (3) examples of preclinical and clinical studies using this delivery method; and (4) recent progress of further improvement of this system.

Cationic liposome/lipid formulations

Since the first report by Felgner *et al* in 1987,[15] more than a dozen other cationic liposome/lipid formulations have been described (see Table 1). As shown in Table 1 and Figure 1, there is little similarity in the structures of different cationic lipids. An active cationic liposome formulation is usually small, unilamellar liposomes prepared by sonication or microfluidization.[15] Occasionally, multilamellar liposomes prepared by simple vortex,[19] or dilution of lipid solution from ethanol solvent,[20] are also active. Cationic liposomes normally contain a cationic amphiphile and a neutral 'helper' lipid, dioleoylphosphatidylethanolamine (DOPE). DOPE is required for non-bilayer forming cationic lipids to form stable cationic liposomes; these include cationic cholesterol derivatives,[21,22] lipopolylysine,[23] and some double-chain cationic surfactants.[24] Most double-chain cationic lipids can form liposomes by themselves, or form liposomes as a mixture with DOPE or other lipids.

Table 1 lists the composition and relative transfection efficiency *in vitro* and *in vivo* of various cationic liposome formulations based on the published data. Since there are few direct comparisons between different formulations in the same experiment using an identical cell type, the assessment of transfection efficiencies should be considered as relative. It should also be noticed that for a given liposome formulation there are variations in terms of transfection efficiency on different cell types. Cationic liposomes containing multivalent cationic lipid usually show better transfection activities than those containing monovalent cationic lipids.[20,25] For example, LipofectAMINE (GIBCO BRL, Gaithersburg, MD, USA) is consistently more active in transfection than Lipofectin (GIBCO BRL).[25]

All cationic lipid molecules contain four different functional domains: a positively charged head group(s), a spacer of varying length, a linker bond and a hydrophobic anchor. The head group of most known cationic lipids contains a simple or multiple amine group with different degrees of substitution, with one exception being an amidine group.[26] The amine groups range from primary amine to quaternary ammonium with substitution of methyl or hydroxyethyl groups. In some cases several different

Table 1 A survey of currently available cationic liposome formulations

Cationic liposome	*Composition*	*Manufacturer*	*Probable biodegradability of cationic lipid*	*Transfection activity*[a]		*Reference*
A. Commercialized				*In vitro*	*In vivo*	
Lipofectin	DOTMA/DOPE=1:1 (w/w)	GIBCO BRL	No	+++	++	(15,16,66)
DOTAP	DOTAP	Boehringer Mannheim	Yes	+++	++	(28, 61)
TransfectAce	DDAB/DOPE=1:3 (m/m)	GIBCO BRL	No	+++	NA	(24)
LipofectAMINE	DOSPA/DOPE=3:1 (w/w)	GIBCO BRL	Partial	++++	NA	(25)
Transfectam	DOGS	Promega	Partial	++++	+++	(20, 80)
B. Not commercialized						
CTAB	CTAB/DOPE=1:4 (m/m)		No	++	NA	(92)
C_{12}GluPhCnN$^+$	C_{12}GluPhCnN$^+$		Yes	+++	NA	(27)
C_{12}GluCnN$^+$	C_{12}GluCnN$^+$		Yes	+++	NA	(27)
Lipopolylysine	Lipopolylysine/DOPE=1:8 (m/m)		Yes	+++	NA	(44)
Cationic cholesterols	Cationic chol/DOPE=1:1 (m/m)			Yes	+++	NA (21)
DC-chol	DC-chol/DOPE=3:2 (m/m)		Yes	+++	+++	(22)
DMRIE	DMRIE/DOPE=1:1 (m/m)		No	+++	+++	(19, 59)
DOTMA/chol	DOTMA/cholesterol=1:1 (m/m)		No	+++	NA	(64)
Lysyl-PE	Lysyl-PE/β-alanyl cholesterol=1:1 (m/m)		Yes	+++	NA	(29)(52)

[a]Since data for direct comparisons are not available, the assessment is relative and semiquantitative.
NA, not available

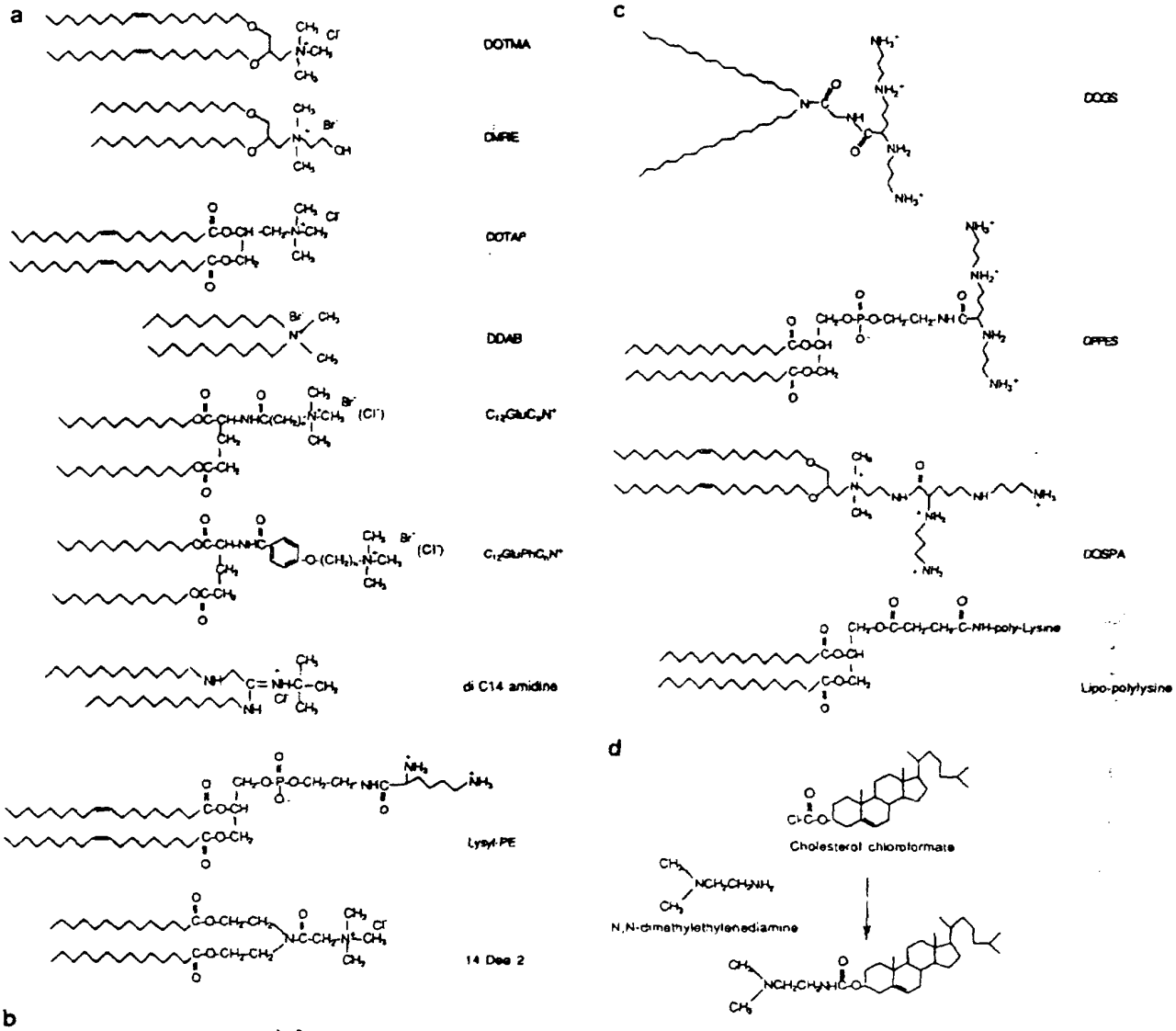

Figure 1 (a) Structures of double-chain, monovalent cationic amphiphiles (b) Structures of cationic cholesterol derivatives. (c) Structures of multivalent cationic amphiphiles. (d) Synthesis scheme of 3β[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol (DC-chol)

types of amino groups coexist in a single cationic lipid (dioctadecyldimethylammonium chloride (DOGS) and 2,3-dioleoyloxy-N-(2(sperminecarboxamido)-ethyl)-N,N-dimethyl-1-propanaminium trifluoro-acetate (DOSPA)).[20,25] The number of charged groups varies from monovalent to multivalent.[15,19–30] The head group of a cationic lipid is responsible for interactions between liposome and DNA, and between liposome–DNA complex and cell membrane or other components of the cell. The interaction is vital for the transfection activity and may contribute to the toxicity of the treatment. The importance of choosing a particular type of head group has been demonstrated in our studies with cationic cholesterol derivatives.[21] Cationic cholesterol compounds with tertiary amino groups show more superior transfection activity and less toxicity to the treated cells than their quaternary ammonium counterparts[21] (See next section). In another study by Felgner's group, hydroxyethyl substituted derivatives of N-(2,3-(dioleoyloxy)propyl)-N,N,N-trimethyl ammonium chloride (DOTMA) show improved transfection activity over the parent compound.[19] It has been hypothesized that the additional

hydroxyethyl group enhances membrane hydration and helps to stabilize the liposomal bilayer structure. Cationic lipids with multivalent head groups bind to DNA and form complexes that are more compact than those formed between DNA and monovalent cationic lipids, which may at least partially contribute to the high transfection activities of the multivalent cationic lipids-counting liposomes.[20,23,25]

For some cationic lipid systems, the spacer arm appears to be less critical for the transfection activity. Examples include the cationic lipid dimethyldioctadecylammonium bromide (DDAB), which contains no spacer, and cationic lipids such as 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), DOTMA and their derivatives, which contain only a one-atom spacer. Increasing the length of spacer to five to eight atoms did not provide the analogs with better transfection activity than DOTAP.[28] The studies from our group on the series of functional cholesterol derivatives also indicated that the length of spacer arm can be varied between three and six atoms.[21] On the other hand, a spacer arm is a crucial factor for the transfection activity of certain cationic lipids. For example, a spacer arm is important for a lipid-anchored spermine head group to bind DNA in the minor groove,[31] and for high transfection activity of the lipid.[31,32] Liposomes composed of cationic lipid with longer spacers show enhanced interaction with the surface of mucosal tissue.[29] From the study of a series of cationic glutamate diesters by Ito *et al*, it seems that cationic lipids containing long spacers (11 atoms) are less active than the ones with shorter spacers (two to six atoms).[27]

The linker bond is an important parameter which determines the chemical stability and biodegradability of a cationic lipid. Cationic lipids such as DOTMA and DDAB contain ether or C-N bonds. Such stable linkers provide excellent chemical stability, but they are not likely to be biodegradable in the cell. The safety of cationic lipids containing these stable bonds, for use in humans, is thus questionable, especially for repeated administrations. Cationic lipids with ester bonds are more biodegradable and therefore have less cytotoxicity.[21,28,29] However, cationic lipids with ester bonds as linkers are generally not chemically stable. For example, liposome composed of DOPE and a cationic cholesterol derivative with an ester bond showed a half-life of about one day at 4 °C, which makes it difficult to use practically.[21] Therefore several cationic lipids were designed to contain stable but biodegradable bonds such as amide and carbamoyl bonds.[21,22,27]

Two types of hydrophobic anchors have been used in cationic lipids, one is a pair of aliphatic chains, and the other is a cholesterol ring. The fatty chains are generally monounsaturated (oleoyl, C18) or shorter saturated (C14 or C12) which offer sufficient membrane fluidity and good lipid mixing within the bilayer at physiological temperature.[15,19,27,28] Felgner *et al* have recently shown that as the length of the saturated aliphatic chain was increased from 14 to 16 and 18 carbon atoms, the transfection activity of the resulting cationic lipids progressively declined.[19] On the other hand, lipospermines containing either two palmityl chains (C16, saturated), or two oleoyl chains (C18 monounsaturated) show high transfection activity,[32] suggesting that the physical property of the lipid anchor is less important for multivalent cationic lipids than monovalent cationic lipids.

A cholesterol ring can also serve as a useful hydrophobic anchor because it exists naturally and derivatives with various functional groups are readily available.[21,22,28,29] Cholesterol is known for its capability of keeping lipids in good packing order and thus offers rigidity to the bilayer membrane. Liposomes containing a cationic cholesterol derivative are more stable in the presence of interfering substances such as ions in the culture media,[28] or a negatively charged mucus surface.[29] On the other hand, cationic liosomes with a cationic cholesterol derivative display equivalent, or better fusion activity than that of cationic liposomes containing cationic lipids with double aliphatic chains.[28] A number of the cholesterol based cationic lipids have been reported.[21,22,28,29] Cholesterol derivatives with quaternary ammonium head groups showed weak but detectable *in vitro* transfection activity.[28] Results with this group showed that tertiary but not quaternary derivatives of cholesterol are active cationic lipids.[31] Another derivative with a β-alanine linked to the cholesterol ring via an ester bond showed good activity.[29] We have reported a simple procedure to synthesize a tertiary amino derivative with a carbamoyl bond (3β(N-(N′, N′-dimethylaminoethane) carbamoyl)-cholesterol (DC-chol)), which provides excellent stability and transfection efficiency as well as potential biodegradability, and hence reduced toxicity compared with the widely used cationic lipid with an ether bond.[22]

Although most of the cationic lipids are synthesized on the basis of trial and error, the accumulated knowledge has nevertheless helped us to design better cationic lipids. An example is the synthesis of a novel cationic cholesterol derivative DC-chol. It has a tertiary amino group and a spacer that offers good transfection activity, a stable carbamoyl bond that provides good shelf-life and a possible biodegradability feature. Another feature is that DC-chol can be synthesized by one-step reaction with good yield from dimethylethylenediamine and cholesterol chloroformate.[22] DC-chol/DOPE liposomes actively transfect many different types of cell lines. Its transfection activity and toxicity have been compared with those of Lipofectin in A431 cells, a human epidermoid carcinoma cell line. It showed about two- to three-fold higher transfection efficiency and lower cytotoxicity.[22] Another group has reported that the DC-chol/DOPE liposome is at least as good as that of Lipofectin in other cell types.[33] DC-chol/DOPE liposomes became the first cationic liposome formulation used in human clinical trials because of their combined properties of transfection efficiency, stability and potential safety.[17,18]

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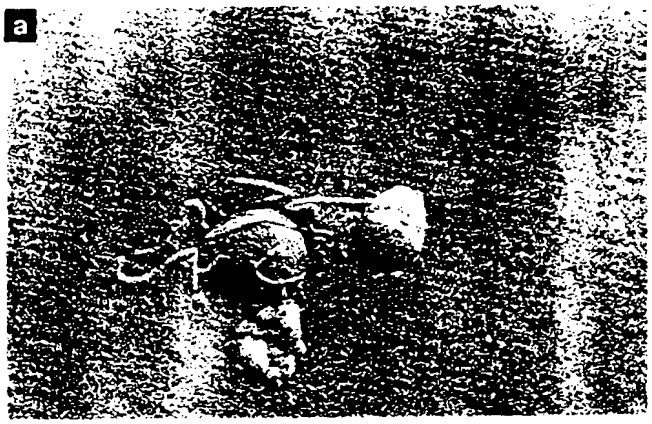
Mechanism of cationic liposome-mediated gene delivery

Much effort has been devoted to the development of better cationic formulations and new applications of this technique. Studies designed to elucidate the mechanism of cationic-mediated liposome gene transfer have been limited. Several approaches are used to study the transfection process. These include: (1) use of a series of structural analogs to elucidate the structure–function relationship of active cationic lipid and helper lipid required for the transfection activity; (2) biophysical and biochemical studies to define the kinetics of complex formation, the structure of DNA–liposome complexes and the interaction between the complex or liposomes and the cell membrane; and (3) morphological and other cell biological studies to monitor the interaction of DNA–liposome complex and cell. These approaches have provided us with some valuable insights into how cationic liposomes transfect cells. Cationic liposomes presumably play three major roles: (1) complexing and condensation of DNA molecules through charge interaction; (2) interaction with cell membranes and triggering uptake of the complexed DNA; and (3) delivering DNA across the cellular membrane into the cytoplasm. For a successful transfection, additional interactions between cellular components and cationic liposome–DNA complex must take place in order to free the DNA from the complex and allow the DNA eventually to enter into the nucleus. Alternatively, the complex enters into the nucleus and the DNA is then uncoated for transcription.

The structural features of the DNA–liposome complex have not been fully revealed. It has been assumed that cationic liposomes bind to the DNA and maintain the original size and shape.[34] Mode of interaction between DNA and cationic liposomes DOTMA and DOTMA/DOPE was studied by Gershon *et al*.[35] Results from fluorescence quenching assay using a base chelating dye indicate that upon cationic liposome binding, the conformation of DNA dramatically changes in a cooperative fashion at a charge ratio of positive to negative slightly over one. There is an extensive membrane fusion or lipid mixing between liposomes, indicating that massive reorganization of the lipid structure occurs during the process. Electron microscopy (EM) studies have revealed that DNA is collapsed into various condensed structures during the process, eventually becoming a rod-like structure apparently encapsulated by a fused membrane tube.[35] Recently, the ultrastructure of DNA–DC-chol/DOPE liposome or DNA–Lipofectin complex has been studied by freeze-fracture EM. The results indicated that the structure of the complex was heterogeneous and was quite different from both models mentioned above.[36] Aggregated and fused liposomes, together with tubular 'spaghetti-like' structures are abundant (Figure 2a). It has been proposed that the tubular structure represents a single supercoiled DNA rod covered by a single membrane of lipid bilayer (Figure 2b).[36] Whether any of these structures is responsible for the transfection activity is not known at present.

Both cationic liposomes and simple cationic polyamines can mediate DNA complexing and condensation as well as promoting cell uptake. While some cationic liposomes can transfect cells, most of the cationic polymer alone, with the exception of cascade polymer,[37] can not transfect cells efficiently. Efficient transfection is only seen when the cells are treated with lysosomotropic agents,[38] osmotic shock,[39] or endosome rupture agents such as inactivated adenovirus and synthetic fusogenic peptides derived from enveloped virus.[14,40] Apparently, cationic liposomes are equipped with an intrinsic fusogenic activity which allows a portion of the DNA–liposome complex to cross the cell



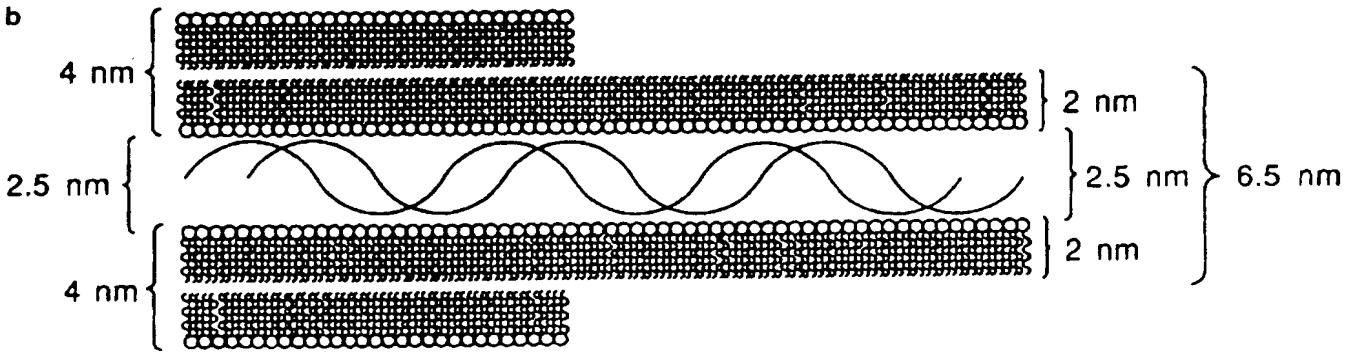


Figure 2 (a) Freeze-fracture electron micrograph of DNA–DC-chol/DOPE liposome complex. A 'spaghetti and meatball' complex is shown. (from Reference 36 with permission). (b) Model of a 'spaghetti'-shaped DNA–lipid complex. (reproduced from Reference 36 with permission from the author, L Huang)

membrane barrier. Otherwise the complex is destroyed in the lysosome when it is internalized by endocytosis. Rapid and large-scale membrane fusion has been observed between cationic liposomes and anionic liposomes,[41,42] especially when DOPE is a major component of cationic liposomes.[15,28,42] It has been suggested that fusion between liposome and plasma membrane could be the major mechanism by which cationic liposome delivers DNA into cells. However, it was found that fusion between DNA–cationic liposome and negatively charged membrane was significantly reduced in comparison with what was observed between free cationic liposomes and anionic liposomes, thus questioning the previous fusion hypothesis.[28] The detailed mechanism by which cationic liposomes deliver DNA is still not fully understood. We have hypothesized that there are two possible mechanisms that could contribute to fusion or disruption of the cellular membrane by DNA–liposome complex. First, for those liposomes containing DOPE, it is this neutral lipid that provides the membrane fusion activity for cationic liposomes. DOPE is a lipid which contains a relatively small head group and two bulky fatty acyl chains which give the molecule an inverted cone shape. It is not favorable to the formation of a stable bilayer structure by itself under physiological conditions. Pure DOPE or membrane enriched with DOPE has a strong tendency to form an inverted hexagonal phase, a structure frequently seen in regions where membranes fuse with each other.[43] During transfection, strong ionic interaction between liposome membrane and plasma membrane may lead to phase separation of the cationic lipids in liposomes. This might generate domains that are enriched with DOPE and promote hexagonal phase transition of the liposome membrane and trigger membrane fusion or disruption. The hypothesis is supported by the fact that several cationic liposome systems require DOPE for their transfection activity. Replacing DOPE with dioleoylphosphatidylcholine (DOPC), a bilayer forming lipid analog of DOPE, severely reduces the transfection activity of cationic liposomes.[15,21,44,45] Zhou and Huang have demonstrated, by using lysosomotropic agents and morphological studies, that the majority of the DNA complexed with lipopolylysine/DOPE liposomes are taken up by cells through an endocytosis mechanism by coated pit and noncoated pathways. The complexes of DNA and DOPE containing liposomes are released from the endosome compartment to the cytoplasm much more frequently than the complexes formed between DNA and DOPC containing liposomes.[44] The hypothesis that DOPE provides the necessary fusion activity seems to be also true for DC-chol/DOPE liposomes as well. Farhood *et al* have recently demonstrated that liposome-mediated transfection can be dissected into three steps,[46] ie binding of the complex to the cell membrane, endocytosis by the cell, and cytoplasmic release of the complex from the endosome. DNA complexing, cell binding and subsequent uptake by cells via endocytosis are dependent on the content of positive charge of the liposome, but not on the composition of the helper lipid. Both DC-chol/DOPE liposomes and DC-chol/DOPC liposomes can mediate these processes. However, the efficiency of the cytoplasmic release in the second step is determined by the DOPE content of the liposomes. The role that DOPE plays in this step can not be substituted by DOPC. Furthermore, cytoplasmic release of the initially bound complex can be facilitated by the addition of free 'helper' liposomes enriched with DOPE which presumably co-enter the cells with the DNA–liposome complex into the same endosome compartment.[46] Although these observations have indicated the crucial role of DOPE as a critical ingredient in cationic liposomes, direct evidence, such as observation of any hexagonal phase formation at the site of endosome membrane fusion or disruption, is still needed to confirm the hypothesis. The second mechanism involving DNA transfection by cationic liposomes that do not contain DOPE, such as pure DOTAP or DOGS, could be local membrane destabilization due to lipid exchange and the detergent effect of cationic lipid as suggested by Zhou and Huang[44] and by Felgner *et al*.[19]

The transfection activity and cytotoxicity of cationic liposomes can also be determined by the nature of the cationic lipid. Studies from our group on the cationic cholesterol derivatives showed that the positively charged head group is critical for the toxicity and transfection activity of these compounds.[21] Some cationic amphiphiles are potent inhibitors of protein kinase C (PKC).[47,48] Protein kinase C is a vital enzyme that plays an important role in signal transduction and gene regulation. It has been shown that a PKC activator can enhance calcium phosphate-mediated transfection,[49] a process that also involves phagocytosis or endocytosis,[50] suggesting that this enzyme may also be important in cationic liposome-mediated transfection. To search for cationic lipids that are safe to use and efficient in transfection, a series of cationic cholesterol derivatives that differ only in the head group structure have been synthesized.[21] The ability to inhibit PKC, cytotoxicity and transfection activity of liposomes containing these derivatives has been evaluated. The results show that tertiary ammonium derivatives are four- to 20-fold less potent in PKC inhibition by comparison with the quaternary ammonium analogs. Cationic liposomes composed of an equimolar mixture of DOPE and tertiary amine derivatives are less toxic to the cells than liposomes composed of DOPE and quaternary ammonium derivatives. The transfection activity of cationic liposomes containing tertiary amine derivatives are 10- to 30-fold higher than those containing quaternary ammonium derivatives. The superior transfection activity of tertiary amine compounds might be due to their lower PKC inhibitory activity, or alternatively, less potent in stabilizing DOPE into the bilayer structure as compared with the quaternary amine derivatives. DC-chol, a tertiary amine derivative of cholesterol, has a weak PKC inhibitory activity (K_i > 1 mM),[51] and is efficient in transfection.[22]

Choosing the correct ratio of DNA to liposomes is important for a successful transfection. The ratio of DNA to liposomes determines the particle size and surface charge of the complex. All of these parameters may influence the outcome of a transfection experiment. It is generally believed that under optimal ratio of DNA to liposomes, the surface of the complex contains a net positive charge for efficient binding and uptake by cells. In the presence of serum, such DNA–liposome complexes are much less active, possibly due to charge neutralization of the complex by binding with negatively charged serum proteins.[21] Recently, Debs' group has reported a modified transfection protocol for CV-1 cells that allows transfection in the presence of serum.[52] A lower ratio of liposome to DNA was used to prepare complexes that are active under these circumstances. It is interesting to note that this strategy also worked for several suspension cultured cells *in vitro*,[53,54] as well as *in vivo* application of aerosol delivery of plasmid DNA–liposome complex.[55] It is not clear why complexes formed at low ratios should transfect cells, because these complexes should theoretically carry a net negative charge, and therefore be taken up by the cells less efficiently. One possible explanation could be that liposomes bind and condense DNA in a cooperative manner, so that there may be an uneven distribution of liposomes among the DNA molecules. Only a portion of the total DNA is complexed with liposomes and presumably exists in active form (Felgner, personal communication). Alternatively, only a portion of a given DNA molecule is condensed with lipids. Another possibility could be that the resulting complexes are taken up by cells with a receptor mediated mechanism. It has been shown that mammalian cells express surface receptors for DNA, although it is less clear whether this receptor exists in all cell types.[56]

It is important that DNA–liposome complexes are made in small particle size. For most attached cell lines, DNA–liposome complexes can be taken up by cells via coated pit and noncoated pathways.[44] Both types of endocytic vesicle are limited in the size of particle that can be taken up. Liposomes greater than 200 nm in diameter are not efficiently taken up by the coated pit pathway. On the other hand some cultured cells also have the capability to take up particles of much larger size by a phagocytosis process.[57] Different cell types may differ in their ability to take up particles of different size, which may partially explain why some cells are more difficult to transfect than others. The particle size of DNA–liposome complex varies, depending on the particular cationic liposome formulation, the charge ratio between liposome and DNA, and the final concentration of DNA–liposome complex. At the appropriate ratio, formulations which exhibit the fluid membrane property and/or multivalent head groups such as DOGS,[58] LipofectAMINE (unpublished) and lipopolylysine/DOPE form smaller particles.[44] Liposome composed of DOPE and DMRIE, a derivative of DOTMA that has a hydroxyethyl substituted quaternary ammonium head group and myristyl chains, forms a small complex at low liposome-to-DNA ratios and high concentrations.[59] The additional hydroxyl group is thought to give the liposome membrane a more hydrophilic surface and may reduce the chance of aggregation between particles of DNA–liposome complex, thus producing small sized particles.

Cationic liposomes in animal models and clinical trials

Due to its relative simplicity, efficiency and reproducibility, cationic liposome mediated transfection has been widely used to introduce foreign genes into a variety of primary and cultured cells. The use of this class of reagent has been extended to *in vivo* gene transfer applications, such as topical applications of DNA–liposome complex to lung[16,60] and nasal epithelium,[18] by aerosol,[55,55,61] arterial endothelium by catheters,[62] direct injection into brain,[63,64] and tumors,[17,65] or by systemic administration.[16,66] DC-chol/DOPE liposomes and DMRIE/DOPE liposomes have been used, or been approved for use, in gene therapy clinical trials in the USA and the UK.[17,18] In this section we will concentrate on the related *in vivo* works and results of the clinical trials.

Intratumor injection of DNA–liposome complex

The first clinical trial using cationic liposome-mediated gene delivery was initiated by Nabel *et al*.[17] The purpose of the trial was to introduce a foreign gene that codes for an allogeneic class I major histocompatibility complex (MHC) antigen into tumor cells *in situ* to stimulate the host cytotoxic T-lymphocyte (CTL) response against the genetically modified tumor cells. An important finding from preclinical studies using tumor-bearing mice is that during the process of developing CTLs against the alloantigen, the host also generates a tumor specific CTL response resulting in a therapeutic effect in the tumor bearing mice.[65] Accumulation of various immunoresponding cells and high local concentration of cytokines might be the mechanism for stimulating a tumor specific CTL response. This observation provides the scientific basis for a clinical trial for the treatment of metastatic tumor using intratumoral injection of a cDNA that codes for a human allogeneic class I antigen.

Cationic liposomes were chosen as gene delivery vector for several reasons. First, since only transient expression is needed to prime the CTL response, delivery of a nonintegratable, nonreplicating plasmid DNA provides a safe and sufficient method for the purpose. Second, direct injection of DNA–liposome complex into the tumor is a convenient, efficient and reproducible method of gene transfer. Finally, cationic liposomes are nonimmunogenic, and therefore safe for repeated injections if necessary. DC-chol/DOPE liposomes were used because of their low toxicity and high transfection efficiency *in vivo*.

Extensive toxicity studies of DNA–liposome complexes have been performed in mice by Stewart *et al*.[67] Biodistribution of the complex administered through intravenous injection or intratumor injection is followed by quantitative polymerase chain reaction (PCR), and treatment related toxic effect is monitored by pathological, electrocardiographic and clinical biochemical studies.[63] The results indicated that plasmid DNA was consistently detectable in the injected tumor. Occasionally, the complex escaped from the injection site and was detectable in organs outside the tumor. However, no major abnormalities, inflammation, or alteration in liver function, electrocardiograph and serum level of tissue specific enzymes was observed in the treated animals even after intravenous injection with a dose used for the intratumor injection, indicating that the procedure is safe.[67] To test the efficiency, H-2K^b tumor bearing mice were treated by direct injection of a plasmid containing the β-galactosidase reporter gene or a gene encoding H-2K^b murine class I MHC antigen. In tumors injected with β-galactosidase reporter gene, 1–10% positive cells were found at or around the injection site. Tumors injected with foreign MHC gene were found to express the alloantigen; both allo-specific and tumor specific CTL responses were detected in the splenic cells of the treated mice. Statistically significant prolongation of the survival time was observed in the treated animals.[63]

These encouraging toxicity and efficacy data have led to the approval of a phase I/II clinical trial protocol treating five HLA B7 negative patients with advanced melanoma using the HLA B7 gene complexed with DC-chol/DOPE liposomes. A single cutaneous lesion from each patient was treated with three injections of different amounts of DNA–liposome complex. One of the patients was also given an additional injection at a different dose, and, later, a pulmonary catheterization treatment on another deep-seated metastatic lesion. The plasmid was not detected in the blood by PCR at any time after injection. No antibody to DNA was found. The expression of HLA B7 gene product was detected in biopsies from all five treated patients by immunostaining against the antigen or by reverse transcriptase PCR. Both B7 specific and tumor specific CTLs were detectable in at least two patients; one of them responded well to the treatment. The treated lesion as well as several other lesions at distant sites of this patient displayed complete regression. A distant lesion which did not respond to the treatment was later confirmed as a different type of tumor. An independent treatment targeted to the lesion by using a catheter was effective in reducing the size of the treated lesion. The results from this small clinical trial demonstrated the feasibility, safety and therapeutic potential of a direct gene transfer approach using cationic liposome–DNA complex in humans.[17] The trial is also significant because it was the first *in vivo* gene therapy protocol approved for human use. It was also the first trial using a nonviral vector as the gene transfer vehicle.

Recently, Nabel and co-workers reported that DMRIE/DOPE liposome was a better cationic liposome formulation for the purpose of intratumoral injection.[59] DMRIE/DOPE liposomes form complexes of small particle size with DNA at high concentrations, which allows a substantially large quantity of plasmid to be introduced into the tumor. At the elevated DNA dose, the outcome of treatment using this new liposome–DNA complex was better than that of DNA–DC-chol/DOPE complex.[59] Currently a phase I/II, multicenter trial is in progress using DMRIE/DOPE liposomes and HLA B7 cDNA.

Son and Huang have recently demonstrated that human ovarian carcinoma cells (2008 cell line) grown as subcutaneous solid tumors in severe combined immunodeficiency (SCID) mice can be transfected by direct injection of DNA–liposome complex into the tumor.[68] The DNA–DC-chol/DOPE lipid complex formed at the ratio of 1 μg DNA to 10 nmoles lipid was previously shown to be optimal for *in vitro* transfection and the same ratio was used for the previous preclinical studies and human clinical trials. Results from that study shows that for direct tumor injection, however, DNA–liposome complex formed at a low ratio of DNA to liposome (1 μg DNA per nmole lipid) transfected much better than the complex formed at a high ratio of DNA to liposome. More interestingly, the efficiency of transfection by direct intratumor injection of DNA–liposome complex could be significantly enhanced (10- to 20-fold) by intraperitoneal injection of the tumor bearing animals with an anticancer drug, cisplatin, a week before the injection of DNA–liposome complex. The sensitization effect is unique to cisplatin, as transplatin and carboplatin, as well as several other unrelated anticancer drugs, were not active. The sensitization effect was only transient; it peaked at 1 week after the injection and declined thereafter. The tumor appeared to be the only target that responded to the cisplatin treatment; transfection efficiency of other organs such as skeletal muscles was unchanged after the cisplatin treatment. Although the exact mechanism of sensitization has not yet been well characterized, this phenomenon may relate to a series of events triggered by cisplatin. Cisplatin causes damages to DNA and proteins.[69] Cells respond to the damage by increasing DNA repair activity,[70] intracellular anti-oxidant level,[71] and the elevated levels of gene expression as a result of PKC activation.[72] These alterations may result in enhanced delivery of DNA–liposome complex by the cells or elevated expression of the transgene in the cells. This observation indicates that a combined protocol of chemotherapy and liposome-mediated gene therapy may be a useful approach for cancer therapy.

Application of cationic liposomes in airway and systemic gene delivery

Because the lung has a large epithelial surface to which it is relatively easy to gain access through the airway, and also because there is a significant proportion of the population that is suffering from

inherited or acquired lung diseases, gene therapy targeted to lung has gathered much attention in recent years. Adenoviral vector,[6,73] and more recently adeno-associated virus vector,[74] have been used successfully to deliver reporter and therapeutic genes such as cystic fibrosis transmembrane conductance regulator (*CFTR*) gene[73,74] and human α-1 antitrypsin gene to the lung.[6] Since there is an increasing concern about the host immune response to adenovirus, which renders the repeat use of this viral vector difficult, nonimmunogenic and noninvasive vectors such as cationic liposomes become an important alternative. The feasibility of gene delivery by cationic liposomes through the airway has been demonstrated using reporter genes in mice,[16,33,55,61,75] rats,[60,76] and rabbits.[77] Various *in vivo* transfection experiments have been successful, especially those via intratracheal instillation and aerosol inhalation. When a reporter gene was delivered with Lipofectin to mice,[16] or rats[60,76] through airway, a significant level of gene expression was detected over a period of 1–7 days. More recently Debs' group has shown that prolonged gene expression (21 days) can be achieved by using DOTMA/DOPE liposomes–DNA complex delivered with aerosolization. Gene product was found in both bronchiolar and alveolar epithelial cells.[55] Other liposomes such as DC-chol,[33] and DOTAP[61] can also mediate efficient transfection in the airways of experimental animals. Both Brigham's and Debs' groups have reported that a significant level of transfection can be achieved by a direct intravenous injection of DNA–liposome complex using Lipofectin or DOTMA/DOPE liposomes.[16,66] Significantly high levels of reporter gene expression were detected in the lungs and other major organs excluding the brain.[66]

A potential application of lung gene therapy is to correct the phenotypes resulting from a genetic disease such as cystic fibrosis (CF). Cystic fibrosis is inherited as an autosomal recessive disorder, and is caused by mutations of a single gene coding for CFTR. CFTR is a cAMP-regulated chloride channel which is required for ion and water movement across the epithelial cell layers. Defective CFTR function leads to respiratory and intestinal disorders.[78] Delivery of the wild-type *CFTR* gene to the diseased epithelial cells offers an opportunity to correct the phenotype of cystic fibrosis.

Following the success of delivering the *CFTR* gene by cationic liposomes and restoring the chloride channel activity in transgenic CF mice,[33] a phase I clinical trial was initiated at the National Heart and Lung Institute in the UK. The trial has recruited 15 CF patients. DC-chol/DOPE liposome–DNA complex was applied via a nasal drip over a period of time. Toxicity to the nasal epithelium, signs of inflammation and efficiency of the gene transfection were followed by morphological examination and reverse transcriptase PCR on nasal mucosa obtained from biopsies. The functional activity of the chloride channel was also analyzed by measuring the membrane potential difference in the nasal epithelium, before and after the treatment. Initial results indicated that this procedure is safe and demonstrates no treatment related toxicity. Furthermore, a statistically significant, partial restoration (approximately 20%) of the membrane potential difference deficit in the treated individuals was observed.[18] The next step will be to use nebulized aerosols to deliver DNA–liposome complex to the lung.

A similar study using DMRIE/DOPE and DOTMA/DOPE liposomes on experimental animals has been published recently.[76] Human *CFTR* cDNA and *lac Z* reporter gene were delivered as DNA–liposome complex into the airways of rats and rabbits using direct instillation. In contrast to the previous protocol for the clinical trial, a much lower lipid-to-DNA ratio (1:8 w/w) was used. Significant levels of *lacZ* gene expression were observed in over 50% of the bronchioles in lung sections. About 70% of the surface epithelial cells were stained positive for the reporter gene product. Other genes including human *CFTR* cDNA were also successfully introduced into the airway epithelial cells in the rats. A 5.2-fold elevated chloride secretion rate was observed in the transfected tracheal cells *versus* those of the control animals. Reverse transcriptase PCR results confirmed that there was specific mRNA synthesized in the transfected airways in the treated animals. A relatively large dose of DNA (4 mg per lung) was used for each animal. The treatment caused some infiltration of mononuclear cells with small foci of the lung, but the inflammation appeared to be mild to moderate and transient (lasting about 10–21 days). No long-term pulmonary or extrapulmonary inflammation was noticed. It was not clear whether DNA, liposome or the combination of both was the cause of the inflammation.[76] These results suggest that an efficient and relatively mild gene transfer procedure using DNA–cationic liposome complex could be used for the treatment of cystic fibrosis in humans.

Besides major efforts in lung gene delivery, two groups have recently reported successful gene transfer to other parts of the body after systemic administration of DNA–lipid complex. The first group demonstrated tumor growth reduction and regression in nude mice carrying tumors derived from a human breast cancer cell line after injection intravenously with the wild-type human p53 cDNA complexed with DOTMA/DOPE liposomes.[79] The treatment also reduced the tendency to relapse and metastases of these tumors. Histological examination revealed reduced mitotic events, infiltration of lymphoid cells at the periphery, deposit of extracellular matrix material, and increased apoptosis events that exist in a scattered pattern in tumor sections from the treated tumors in comparison with the control groups. The presence of human p53 cDNA was confirmed directly by PCR in treated tumors, and the expression of wild-type p53 protein was demonstrated indirectly by a function assay with the elevated expression of p21 transcripts which is transcriptionally activated by p53. Another group reported that DOGS, a cationic lipospermine, could complex and deliver reporter genes to the fetus in pregnant mice.[80] At a special gestational stage, about

day 9.0 postcoitus, the fetuses could be transfected efficiently by intravenous injection of DNA–lipid complex. DNA was found in the episomal form from major organs, such as heart, lung, liver and skeletal muscle up to 40 days after the injection from 1-month-old newborn mice. The expression of the reporter genes was demonstrated by Northern blot, enzymatic assay of tissue extracts and histochemical staining. The gene expression appeared to be transient but widespread in the fetal tissues. The authors mentioned that weak but detectable expression existed up to 1 month after birth. This treatment appeared to be safe for both dams and the progeny. The transgenes were not passed-on to the next generation from the gene-transferred mice which developed normally. This new system provides a simple method that allows the delivery and expression of a transgene into the fetus and may have important implications in gene therapy.

Problems and future development: molecular biology meets chemistry

Several urgent problems need to be resolved concerning *in vivo* applications of cationic liposome-mediated gene delivery. The most important issue is how to enhance the transfection efficiency. Although significant transgene expression has been achieved using this method, the level is relatively low in comparison with what can be achieved with adenoviral vectors, at least in experimental animals. The presence of interference substances in the body fluid such as serum proteins, mucus and surfactant significantly reduces the transfection efficiency. Relatively small particles, not overly positively charged complexes such as the ones used by Debs' group may be more useful than the ones that work optimally for *in vitro* transfection. Research directed towards making better cationic lipids is highly desirable. For example, a formulation that is more resistant to the shear force of the nebulization process and to the surfactant must be advantageous for airway gene therapy. The use of additional amphiphilic peptides has been shown to be beneficial.[81] Improvement in the current formulation of DNA–liposome complex for clinical trials is also needed. In both trials, DNA and liposomes were packaged in separate vials, and mixed at the bed side. A single vial formulation would be more convenient and pharmaceutically acceptable.

A successful transfection is the combination of efficient delivery and gene expression. One can search for a strong expression system by optimizing various steps in gene expression, for example, by including an intron sequence or translational enhancer.[82] Using strategies that can improve the nucleus targeting of the delivered DNA may also increase the expression level of a transgene, since it is known that translocation of DNA from cytoplasm to nucleus is rather inefficient.[83] Alternatively, the nuclear targeting issue can be overcome by developing a more efficient cytoplasmic expression system, such as the one that uses T7 RNA polymerase/T7 promoter as a potent expression system.[45,84–86] The concept is illustrated in Figure 3.

Another major problem with this delivery system is the lack of target specificity. This problem has been considered by our group and others.[87–89] One way to achieve target specificity is to incorporate targeting ligands such as antibody and asialoglycoproteins into the DNA–liposome complex. Our group has proposed the 'ternary complex' concept illustrated in Figure 4 which contains a plasmid, cationic liposomes and modified target ligands that can bind to the DNA. To test the feasibility of the concept, polylysine of low molecular weight is conjugated to a rat monoclonal antibody against a surface antigen on mouse endothelial cells. Polylysine–antibody conjugates bind to DNA and the rest of the charges on the DNA are then

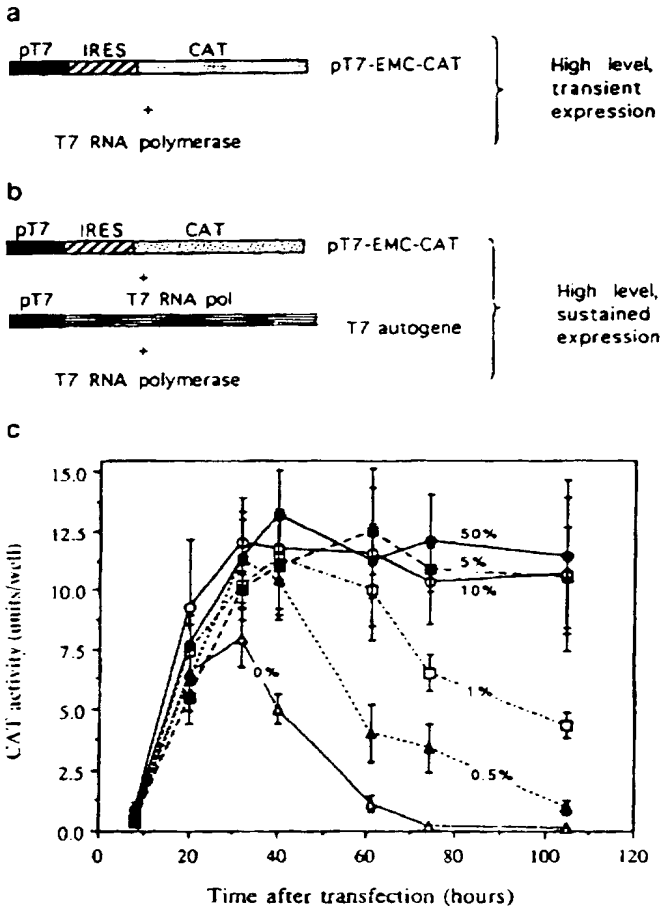
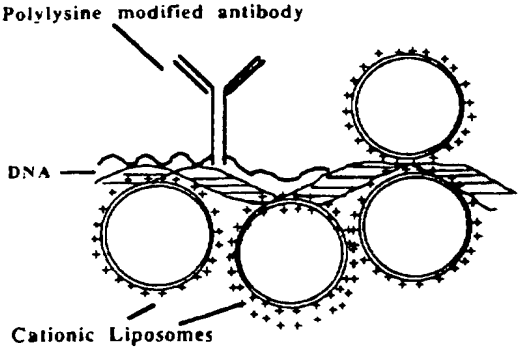


Figure 3 (a) Strategies of cytoplasmic expression of a reporter gene driven by a T7 promoter via codelivered purified T7 RNA polymerase (a), or purified T7 RNA polymerase and a T7 RNA polymerase autogene which regenerates T7 RNA polymerase inside the cell (b). pT7, T7 promoter, EMC, internal ribosome entry sequence of encephalomyocarditis virus, CAT, chloramphenicol acetyltransferase; T7 RNA pol, T7 RNA polymerase gene. (c) Time course of CAT gene expression with or without T7 autogene. 293 Human embryonic kidney cells were transfected with DC-chol liposomes and DNA composed of pT7-EMC-CAT and various amounts of T7 autogene as indicated. To initiate the transcription, 150 U T7 RNA polymerase was included. (Modified from Reference 84 with permission from the publisher, Academic Press, Orlando, FL, USA)



DNA/Cationic Liposome/Antibody-PLL Ternary Complex

Figure 4 Schematic representation of ternary DNA/cationic liposome/antibody-PLL complex. +(Modified from Reference 87 with permission from the publisher Elsevier Science, Amsterdam, The Netherlands)

neutralized by cationic liposomes which are added simultaneously with the polylysine–antibody conjugates. In the ternary complex, the antibody provides target specificity and may trigger endocytosis of the complex. DC-chol/DOPE liposomes presumably provide the endosome rupture capability and help the release of DNA into the cytoplasm. Preliminary results indicated that such a ternary complex specifically bound and transfected mouse lung endothelial cells *in vitro*,[87] and the DNA was targeted to the mouse lung *in vivo* in a specific manner.[88] A similar approach has been tested by Zeldis and co-workers.[89] Polylysine conjugated asialoglycoprotein was used as a specific ligand for the asialoglycoprotein receptors on the hepatocytes, and DOGS cationic lipid was used to facilitate release of the complex from the endosome compartment. At appropriate ratios of liposome-to-target ligand, HepG2, a hepatoma cell line which expresses asialoglycoprotein receptors, was transfected efficiently and specifically.[89] Adenovirus and other endosome rupturing peptides have been used recently to increase the efficiency of intracellular delivery of target specific DNA–ligand-polylysine conjugates.[14,40] None of these new constructs has been extensively tested *in vivo* for targeting specific transfection.

The last but not the least problem shared by all nonviral and some viral vectors is the duration of gene expression. A transient expression in some applications is an advantage but in other situations is a serious drawback. Expression of most transgenes delivered by cationic liposomes lasts only about a week or less, although some have reported longer periods of expression.[66] There are currently two possibilities for improving the expression time. One is to take advantage of certain viral components that facilitate integration of its genome, such as the integratase of retrovirus. Co-introducing DNA and purified integrase has been reported to achieve enhanced stable transformation of the cells in culture.[90] Inclusion of the terminal repeat (TR) sequence from AAV into a plasmid expression vector has been shown to prolong the duration of transgene expression in lymphocytes, and in transformed epithelial cells.[54] Although the mechanism underlying this phenomenon has not yet been revealed, there might be DNA sequences functionally similar to the AAV TR, and specific factors in the host cells which are responsible for the prolonged gene expression. Understanding the mechanism may lead to an important solution to the problem. Preliminary results from our group indicate that prolonged expression of a transgene can be achieved in transformed epithelial cells by co-delivery of the AAV Rep protein with an expression vector flanked by two AAV TR termini. The expression vector alone, however, is not sufficient to sustain the expression in transformed human epithelial cells.[92] Self replicating vectors, such as episomal elements or artificial chromosomal vectors may also be used for long-term expression of a transgene.

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